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Genetic Targeting of a Voltage-Sensitive Dye by Enzymatic Activation of Phosphonooxymethyl-ammonium Derivative

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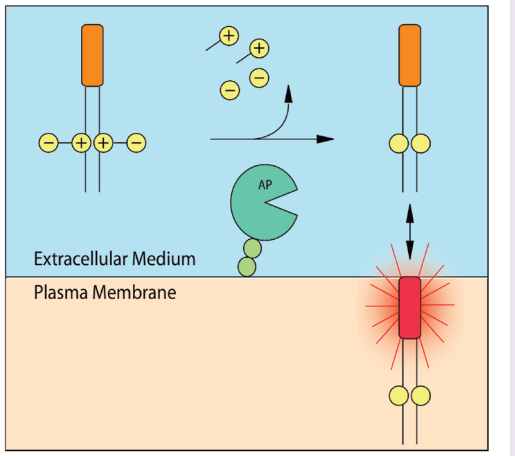
ABSTRACT: Optical recording of action potentials in individual neurons requires cell-selective targeting with a fluorescent, voltage-sensitive probe. We report on a new labeling system that takes advantage of recent developments in prodrug-based chemistry and allows for the targeting of a lipophilic dye into the plasma membrane of genetically specified cells. With the introduction of two phosphonooxymethylammonium zwitterions into the hydrocarbon chains of an amphiphilic, voltage-sensitive hemicyanine dye, a precursor dye was formed that is water-soluble to an extent that it can no longer bind into cell membranes and hence prevents unspecific staining. Glycosylphosphatidylinositol anchored placental alkaline phosphatase expressed in HEK293 cells converted the precursor dye to a homologue of the widely used dye di-4-ANEPPS and gave rise to excellent levels of plasma membrane localized staining. The voltage sensitivity of the enzymatically activated dye was tested and shown to be similar to sensitivity reported for di-4-ANEPPS.



A wide variety of techniques are currently used to investigate the electrophysiology of the brain. The well-established methods, able to monitor single neurons, require the insertion of micropipets or metal electrodes into brain tissue. However, there has been much work on the development of optical methods for the recording of electrical signals,[1] and these methods potentially offer the highest spatial resolution for observing the electrical activity of the brain. Small-molecule fluorescent probes are able to report a signal that is proportional to the change of the trans-membrane voltage during an action potential.[2] Research has been performed on determining the mechanism by which these compounds function,[3] on increasing their sensitivity,[4] and on optimizing for the desired spectral range.[5] The most sensitive compound yet developed, ANNINE-6, has been shown to achieve a fractional fluorescence change up to 50%/100 mV.[6] However, extracellular application of voltage-sensitive dyes to brain tissue leads to the staining of all cell types, preventing the visualization of individual neurons. Thus, staining and optical recording of single neuron activity is only possible with time-consuming and complicated intracellular loading of the dye, which also results in the staining of intracellular membrane.[7]

To overcome the limitations of unspecific staining, two directions have been taken in developing methods for genetically targeting individual neurons. The purely genetic approaches express fluorescent proteins fused to voltage-regulated integral membrane proteins.[8,9] These rely on the voltage-sensitive displacement of trans-membrane protein domains to modulate Förster resonance energy transfer (FRET) or induce transient disruption of fluorescent proteins[10] to generate an optical signal. On the other hand, two hybrid approaches have been proposed, both of which rely on the expression of proteins in the target cells and the addition of a compound into the tissue culture medium. In one system, membrane-anchored GFP is expressed in genetically specified neurons. After the addition of lipid-soluble dipicrylamine, which binds into the cell membrane of all of the cultured cells, voltage sensitivity is achieved *via* the voltage-dependent displacement of the compound within the lipid bilayer, modulating FRET with GFP.[11] In the other system, targeted staining was induced with the enzymatic cleavage of anionic phosphate groups attached to a soluble precursor dye, yielding a membrane-binding, amphiphilic dye.[12] At present, all of these methods have serious shortcomings. The voltage-sensitive proteins exhibit a rather weak and slow voltage-sensitive response.[10] The hybrid approach utilizing anionic dipicrylamine significantly affects the capacitance of the plasma membrane when used at concentration necessary for good signal detection.[11,13] In the hybrid approach with targeted staining, three problems remained unsolved: (i) The ratio of targeted staining to background staining is low as a result of the precursor dye binding to the untargeted cells. (ii) The transformed dye was not shown to be voltage-sensitive. (iii) The method was not applied to nerve cells or brain tissue.

In the present paper, we describe significant progress with the second hybrid approach, with the introduction of a new precursor-dye activation mechanism, together with the incorporation of the

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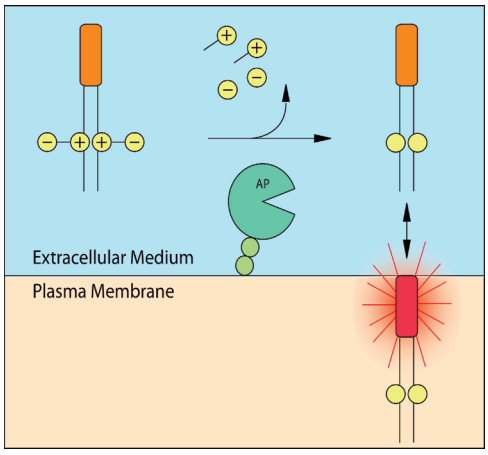

Figure 1. Schematic diagram of the enzymatic activation of a precursor dye to a voltage-sensitive dye in the cell membrane. The precursor dye is water-soluble as a result of zwitterionic groups in the hydrocarbon chains (vertical lines). The chromophore (orange) has a low fluorescence in water. The dye is dephosphorylated by a membrane-bound phosphatase, producing an unstable *N*-hydroxymethyl ammonium intermediate that breaks down to yield an amphiphilic dye that strongly binds to the plasma membrane where the chromophore exhibits a high fluorescence in a hydrophobic environment. The chemistry of the reaction is described in Figure 2b.

chromophore of the widely used voltage-sensitive dye di-4-ANEPPS.[14] Based on chemical techniques used in prodrug design, the solubility of the precursor dye is enhanced so much that its binding to untargeted lipid membrane is completely suppressed. The enzymatically activated dye is tested for voltage sensitivity, with a trans-membrane voltage induced *via* an externally applied electrical field, and is shown to be equal to that of di-4-ANEPPS. The approach is sketched in Figure 1. Zwitterions are introduced in the hydrocarbon chains of the precursor dye, rendering the molecule water-soluble. A membrane-bound enzyme removes the zwitterions. The resulting amphiphilic dye binds to the membrane, with the chromophore located in an unpolar environment.

RESULTS AND DISCUSSION

Voltage-Sensitive Dye Synthesis. Amphiphilic voltage-sensitive hemicyanine dyes bind into the lipid bilayer of the plasma membrane, and their orientation within the membrane is a key factor in their sensitivity.[15] However, such dyes stain the plasma membrane of all cell types, greatly increasing the background fluorescence and thus strongly reducing the detectable signal during optical recording of neuronal activity. In principle, this problem can be overcome by the enzymatic transformation of a water-soluble precursor dye into a lipophilic voltage-sensitive dye that binds to the plasma membrane of a selected cell that expresses a suitable ectoenzyme by genetic targeting.[12] Crucial for this approach, however, is a very high membrane affinity of the active product dye, in combination with a very high water solubility of the precursor dye. Only in such a case can a large voltage-sensitive signal together with a low level of unspecific background staining be expected.

In the development of a novel precursor dye, we took advantage of a prodrug strategy developed for tertiary-amine containing drugs.[16] To overcome poor water solubility of certain drugs, a *N*-phosphonooxymethyl group was attached to a tertiary amine,[17] to form a water-soluble quaternary amine. The active drug was regenerated in a two-step bioreversion process by an enzyme-catalyzed dephosphorylation and then a spontaneous chemical breakdown of the unstable *N*-hydroxylmethyl intermediate. By incorporating this zwitterionic moiety into the aliphatic chains of a voltage-sensitive dye, we aimed to disrupt the precursor dyes ability to enter the plasma membrane.

To develop an enzymatically inducible voltage-sensitive dye, we used the chromophore of the well-known hemicyanine dye di-4-ANEPPS,[14,18] because of the relative ease of its synthesis as compared to the more sensitive ANNINE dye family.[6,19] The crucial steps in the synthesis of the precursor dye are sketched in Figure 2a. At first, an ANEPPS dye with two hexanol substituent was synthesized, following a modified route from Loew.[18] Starting with the easily obtainable 6-bromonaphthalen-2-ol, a functional group interconversion to the amine was performed with Bucherer reaction,[20] with a yield of ∼80%. Alkylation of the newly created amine was initially performed with 6-iodohexan-1-ol in dimethylformamide, which in turn was synthesized from the corresponding bromide *via* the Finkelstein reaction, so as to increase their reactivity with the relatively unreactive aromatic amine. However, the yields for the alkylation of 6-bromonaphthalen-2-amine was found to be around 10%, rather than the reported 70% from the original literature for 1-iodobutane.[18] Further research into the alkylation step showed that the selection of solvent plays a strong role in overall yield, and that the change from the published dimethylformamide to *tert*-butanol increased the yield from ∼10% to 28%.[21]

The alkene bond was constructed with vinyl pyridine, *via* the palladium-catalyzed Mizoroki−Heck reaction,[22] which was chosen for its high yields in the desired *trans* selectivity. The yield published for this step is 77%. However, the actual yield obtained over the course of multiple reactions under the published conditions was an average of only ∼35%. Improvement in the yield was obtained through a modification of reaction solvent. The need of a high-pressure "bomb" as the reaction vessel was eliminated with the addition of a large amount of triethylamine (TEA), with a 2:1 TEA/DMF solution,[23] increasing the yield from ∼35% to 84%.

The headgroup addition published by Loew used the ring opening of 1,3-propanesultone to form the cationic pyridinium betaine. However, the terminal alcohols required for the prodye intermediate would prohibit the use of this reagent without extra alcohol protection and deprotection steps, and so 3-bromopropane-1-sulfonic acid was used in its place.

The synthesis of the *N*-phosphonooxymethyl prodye required the conversion of the terminal alcohols to a good leaving group; unfortunately, the standard tosylation *via* tosyl chloride in either dichloromethane, pyridine, or acetonitrile produced very low yields, due to very poor solubility of the compound in these solvents. Solvent-free mesylation, however, yielded very good results at around ∼90%.[24] The mesylated intermediates were then used to alkylate methyl octylamine, forming the long-chain target dye that binds into the cell membrane.

The soluble precursor dye is obtained by quaternatization with di-*tert*-butyl chloromethyl phosphate and subsequent deprotection.[17] The *N*-phosphonooxymethylation was tested in acetonitrile,[17] and other common aprotic solvents, such as dimethylsulfoxide, dimethylformamide and tetrahydrofuran. Only with acetonitrile, however, was the correct product obtained. The synthesis of di-*tert*-butyl chloromethyl phosphate was initially performed as described in the original paper;[17] however, low yields (∼10%) of the material led us to use another procedure,[25]

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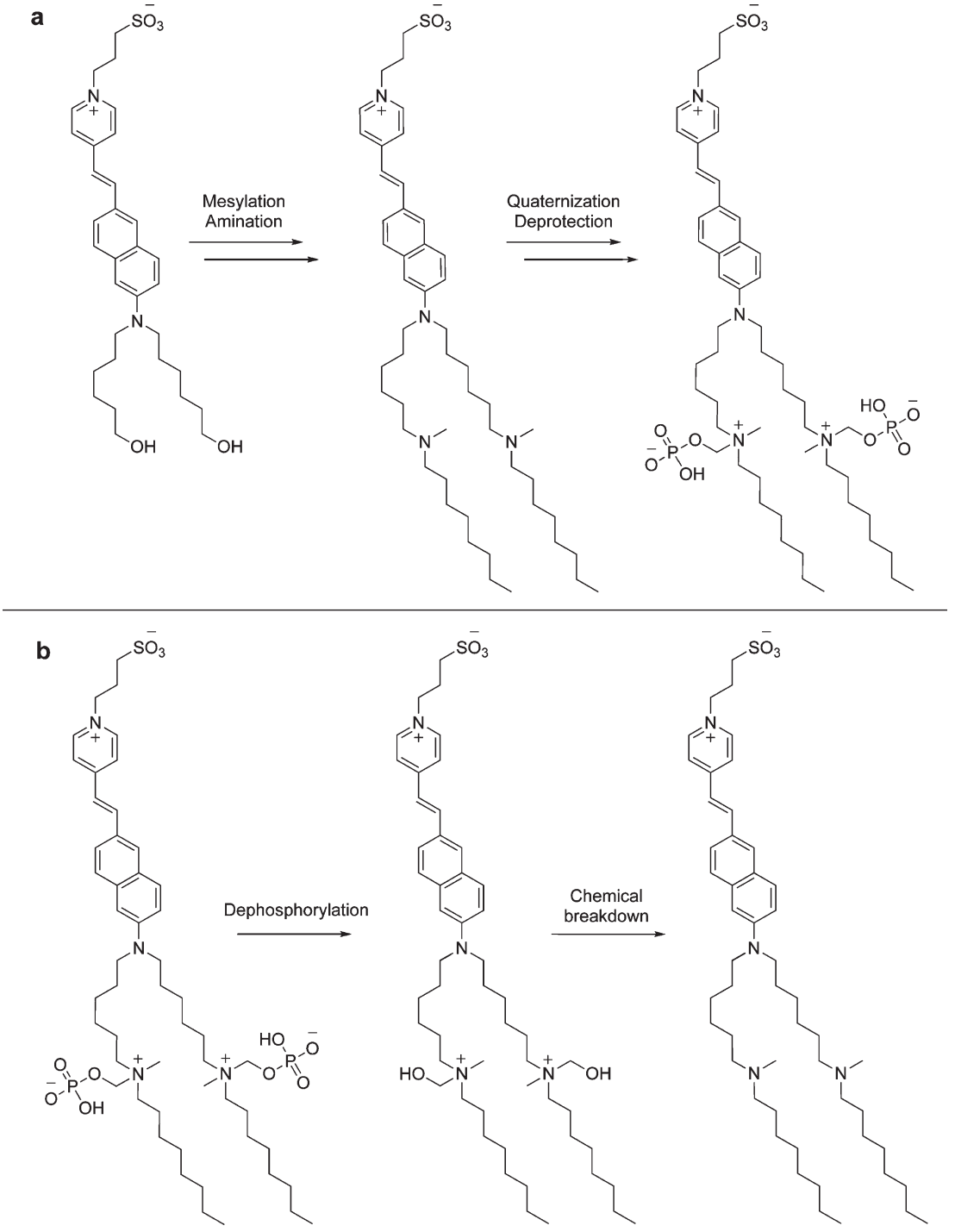

Figure 2. Precursor dye and active dye. (a) Synthesis of the precursor dye, *via* mesylation and subsequent *N*-alkylation of a hexanol analogue of di-4-ANEPPS. This is followed by *N*-phosphonooxymethyl quaternization and deprotection of the dye. (b) Bioreversion of the precursor dye, first by enzymatic dephosphorylation, and then by spontaneous chemical breakdown of the *N*-hydroxymethyl quaternary ammonium intermediate.

giving rise to yields of over 70%. The details of the synthesis are described in the Methods section.

The reaction scheme of the two-step activation is sketched in Figure 2b, with a desphosphorylation and a subsequent breakdown of the unstable *N*-hydroxymethyl intermediate. We tested the activation of the precursor dye in a suspension of lipid vesicles.[26] At pH 8.1, the suspension showed very low levels of fluorescence intensity, corresponding with the low quantum efficiency of the ANEPPS chromophore in aqueous solution, as for other hemicyanines with CC single bonds.[27] After the addition of a water-soluble phosphatase, there was a strong increase of fluorescence as observed in a previous study.[26] This result indicated that the product dye was formed and that the chromophore became embedded in the hydrophobic core of the membrane.[27] A quantitative study of the staining reaction in the model system was not performed.

Extracellular Phosphatase Construction. To observe the targeting of cells with a membrane-bound enzyme, a marker

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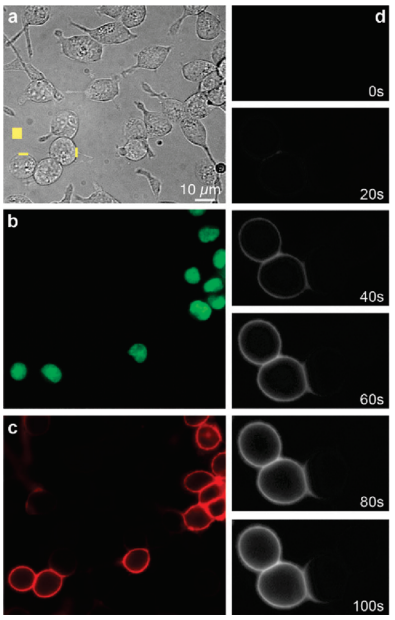

Figure 3. Targeted staining of HEK293 cells with voltage-sensitive dye by activation of precursor dye. (a) Phase contrast micrograph of a mixed population of untransfected cells and cells that are stably transfected with PLAP. The three regions of interest for the measurements of Figure 4 are shown as yellow boxes, marking targeted membrane, control membrane, and background. (b) Fluorescence micrograph that reveals the nuclear GFP labels of cells targeted with PLAP. (c) Fluorescence micrograph that shows the staining of the plasma membrane with the voltage-sensitive dye, 100 s after addition of the precursor dye. (d) Dynamics of staining shown by fluorescence micrographs.

system was developed with the human placental alkaline phosphatase (PLAP). PLAP is a glycosylphosphatidylinositol (GPI) anchored enzyme, located on the outer plasma membrane.[28−30] In the brain, PLAP-expressing neurons show enzymatic activity along unmyelinated and myelinated axons, as well as in dendritic arborizations.[31,32] This localization ensures that an activation of a precursor dye would occur at the entire surface of a genetically specified neuron. This is an important requirement, because voltage-sensitive dyes do not diffuse along plasma membranes. Moreover, stable long-term expression of PLAP has been shown in neurons that were transplanted into the CNS of transgenic rats,[33] and ubiquitous expression of human PLAP in mice showed no adverse effects on mouse development or viability.[34]

To identify cells that are transfected with PLAP, a marker gene was employed; GFP that is fused with the H2B histone is expressed in the cell nucleus.[35] This localization of H2B-GFP provides a spatial separation of fluorescence from the voltage-sensitive dye in the plasma membrane and enables the detection of faint dye signals by fluorescence microscopy, despite an overlap in the emission spectra of the two chromophores. The genes of PLAP and H2B-GFP were expressed on a single vector, with the H2B-GFP gene placed downstream of an IRES element for bicistronic expression. In addition, the vector also contained an FRT recombination site. With the use of the FRT site containing Flp-293 cells, Flp recombinase induced the integration of the vector into the cells in a site-specific manner, generating an isogenic stable cell line.[36] To test for the correct expression of PLAP in this stable cell line, the fluorogenic phosphatase substrate

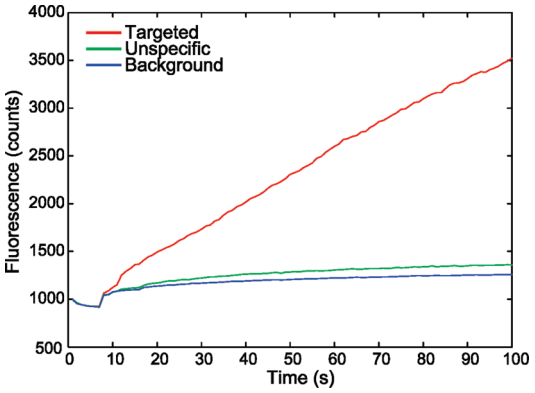

Figure 4. Time-course of fluorescence of the hemicyanine dye during enzymatically activated staining of HEK293 cells. Results are shown for three regions of interest as indicated in Figure 3a, the membrane of a targeted cell (red), the membrane of an untargeted cell (green), and the background of the culture chamber (blue).

ELF-97 was used.[37] No detectable levels of phosphatase activity were found on the surface of untransfected HEK293 cells, whereas the stably transfected cells showed good levels of GFP fluorescence in the nucleus and activation of the ELF97 dye on the cell surface.

Cell-Specific Staining. We prepared a mixed population of HEK293 cells, containing stably transfected cells and untransfected control cells. The protocol for washing, staining, and imaging is described in the Methods section. A bright field image of the cells with a thin film of washing buffer is shown in Figure 3a. The stably transfected cells are identified by fluorescence imaging of GFP as shown in Figure 3b. The staining with the hemicyanine dye was observed by fluorescence micrographs that were taken every second. Eight seconds after the start, 2 mL of staining solution with 1 μM of precursor dye at pH 8.6 was added. Figure 3c shows the fluorescence after 100 s. There is a perfect match between the cells that are stained in Figure 3c and the cells that are genetically targeted as shown in Figure 3b. No significant unspecific staining is observed. A series of fluorescence images in Figure 3d shows the progression of staining over the course of the experiment. The images have been normalized to the highest levels of cell membrane staining, so no remaining GFP fluorescence is visible in the sequence of images. For an extended duration of staining, the last images show that the membrane of a nontargeted cell is stained in an area that is near a phosphatase-expressing cell. This effect is caused by diffusion of the product dye that is present in the aqueous phase near the active cell at a low concentration. The problem of this secondary nonspecific staining has been discussed in ref 27. It can be avoided by further enhancing the binding constant of the activated dye or by limiting the duration of staining.

For a quantitative evaluation of staining we selected three regions of interest, as marked in Figure 3a: (i) the cell membrane of a targeted cell, (ii) the cell membrane of an untransfected cells, and (iii) the cell-free surround in the culture chamber. The fluorescence intensities are plotted in Figure 4 as a function of time. There is a small increase in fluorescence within the first 1−2 s after the addition of the staining solution. It is due to the weak fluorescence of the soluble precursor dye. The background fluorescence slightly increases due to binding of the precursor dye or of activated dye to the glass substrate. The staining of the

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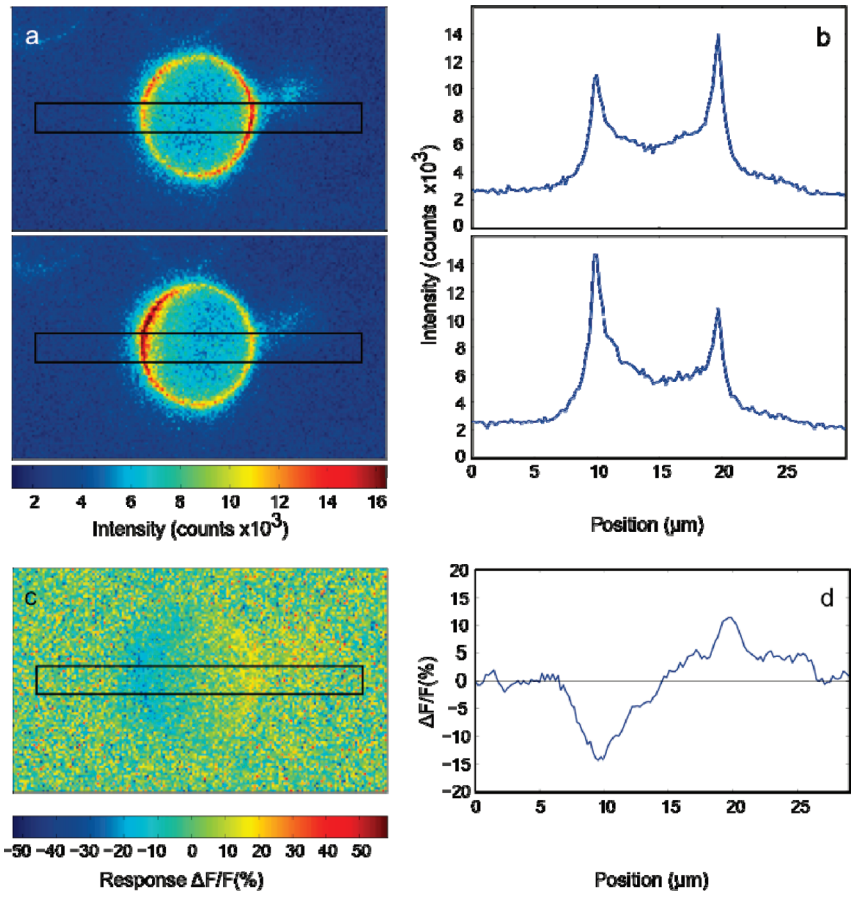

Figure 5. Spatial pattern of fluorescence intensity in a HEK293 cell between two extracellular electrodes. (a) Fluorescence intensity (color coded in arbitrary units) with a positive voltage (26.7 V) applied to the right electrode (upper picture) and to the left electrode (lower picture), respectively. (b) Profiles of fluorescence intensity across the cell as evaluated in the rectangular regions marked in panel a. (c) Relative change of fluorescence intensity $\Delta F/F$. (d) Profile of the relative change of fluorescence intensity.

control cells follows the same pattern, to a level that is marginally higher than the background. The fluorescence of the cell membrane of targeted cells shows an almost linear increase for the first 60 s, followed by a slower increase. After 100 s, the level of fluorescence is more than 25 times higher compared with control cells and background.

Previous work on the selective staining of cell membranes with fluorescent dyes showed excellent selectivity only in lipid vesicles,[26] after the dephosphorylation of two phosphate groups to yield an aliphatic alcohol, leading to increased dye binding strength. However, *in vitro* staining of cells yielded more modest levels of selectivity, yielding a ratio of ~2.5:1 of fluorescence between targeted and control cells.[12] This was due to unspecific binding of the precursor dye to the cell membrane. By replacing the hydrophilic terminal phosphate groups with the strongly charged *N*-phosphonooxymethyl quaternary amine, the precursor dye was found to be dramatically more soluble in water. With the selection of methyl-octyl amine for the compound's synthesis, the precursor dye's zwitterionic charge is placed near the middle of the lipophilic chain. This structure prevents a "folding back" of the aliphatic tail, a mechanism that may cause for the relatively poor selectivity of the precursor dyes with terminal phosphate groups.[12] Unspecific staining is reduced to nearly background levels, while the targeted plasma membrane shows a high fluorescence intensity that is comparable to a staining with 1 μM di-4-ANEPPS.

Voltage-Sensitivity Recording. The voltage sensitivity of the enzyme-activated hemicyanine dye was tested in a chamber with two electrodes of platinum wire.[6] Biphasic square-wave voltage pulses with amplitudes from $\pm$2.67 to $\pm$26.70 V were applied with a duration of 25 ms. Fluorescence images were taken during the positive and negative phase as well as before and after the double pulse. The references before and after the voltage pulses were used to correct for bleaching and as a reference to calculate relative change of fluorescence intensity.

Figure 5a shows color-coded images of the fluorescence intensity of a targeted cell. The wire electrodes are parallel to the vertical sides of the images. In the upper image, the positive voltage is applied to the right electrode. The lower image shows the fluorescence after a reversal of the electrode polarity. In both cases, there is an increase in fluorescence of the membrane that is near the positive electrode. In each image, a region defined by the black rectangle was further analyzed. A vertical integration yielded the fluorescence profiles shown in Figure 5b. Using images before and after the applied voltages as references, we evaluated the pattern of the relative change in fluorescence $\Delta F/F$ as shown in Figure 5c with a profile plotted in Figure 5d.

The voltage drop in the culture medium around a cell arises from the current flow between the electrodes. For a spherical cell with radius r_{cell}, the voltage change ΔV_M across the plasma membrane near the poles of the cell is estimate by eq 1 with the applied electrode voltage V_{el} and the distance d_{el} of the

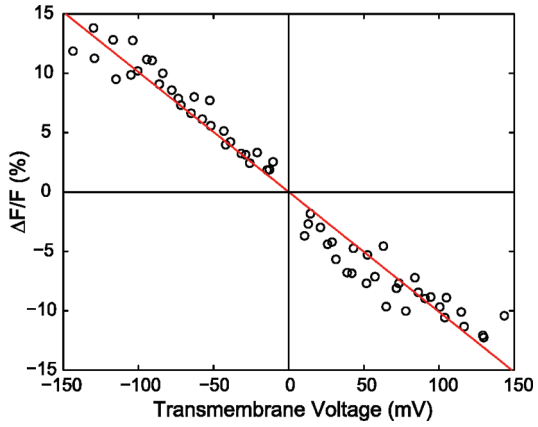

Figure 6. Relative change of the fluorescence intensity $\Delta F/F$ versus change of the voltage ΔV_M across the plasma membrane of a HEK293 cell for a series of voltage pulses applied between two extracellular electrodes. The data are from 4 different cells. The slope of the fitted line indicates a voltage sensitivity of $\Delta F/F\Delta V_M = -10.1\%/100$ mV.

electrodes.[38] This relation remains valid for a hemispherical cell attached to the substrate of the culture chamber.

$$\Delta V_M = -1.5 V_{el} r_{cell}/d_{el} \quad (1)$$

The relative change of fluorescence $\Delta F/F$ is plotted in Figure 6 versus the voltage changes ΔV_M for a series of applied voltages. The data points are combined results from measurements with 4 cells. A linear relation is fitted to the data with a voltage sensitivity $\Delta F/F\Delta V_M = -10.1\%/100$ mV (with 95% confidence bounds of −9.6% to −10.6%). This value is in good agreement with the voltage sensitivity of di-4-ANEPPS.[38−40] The high voltage sensitivity of the enzymatically targeted dye shows that the amino groups in the hydrocarbon chains have no negative effect on the voltage sensitivity, *i.e.*, these hydrophilic groups do not negatively affect the position and orientation of the chromophore in the plasma membrane as compared with di-4-ANEPPS.

At present, the dyes are limited to use at a relatively high pH range; at a pH range between 7.4 and 8.5, the dye performed poorly and showed unspecific binding to dead cells and debris. This effect is due to the protonation of the tertiary amines in the activated dye (∼p*K* 10.3), which prevents binding of the dye to the membrane. However, at pH 8.6 and above, excellent staining is observed. Although this value is higher than physiological pH, neurons can tolerate such high pH for long periods, where it has been demonstrated to protect cells from ischemia; adult rat dorsal root ganglion neurons kept at pH 9.3 at 37 °C for 4 h had a higher viability than those kept in the same conditions at pH 7.6.[41] The higher pH could therefore be briefly used for selectively staining target cell membranes, followed by a return to a lower pH for voltage-sensitive measurements. Further research must be undertaken to modify the basicity of the tertiary amines, to prevent their protonation at lower pH ranges. Such a modification, perhaps by the addition of electronegative functional groups to deactivate the amine, may allow for selective staining at a more physiological pH.

In conclusion, a key limiting factor preventing the use of the hybrid approach for selective staining with a voltage-sensitive dye has been overcome. The new activation mechanism described here allows for dye labeling of genetically targeted cells with very high specificity. This progress has been achieved by introducing zwitterions into the hydrocarbon chains of the precursor dye such that it is binding to the membrane was suppressed. The precursor dye shows exceptionally low levels of background staining, and its high solubility should allow it to deeply penetrate brain tissue. Once activated, the hemicyanine dye shows good voltage sensitivity, at levels equal to that of the widely used di-4-ANEPPS. This technique has the potential to be extended to other fluorescent probes or pharmaceutical compounds, for selective membrane targeting.

METHODS

6-Iodohexan-1-ol. 6-Bromohexan-1-ol (100 g, 735 mmol) and NaI (121 g, 809 mmol) were refluxed in 250 mL of acetone overnight, then cooled, and filtrated. The acetone was removed under reduced pressure, and the mixture extracted with 3 × 100 mL portions of EE, with the combined organic extracts dried over sodium sulfate, filtrated, and concentrated *in vacuo* to yield a thick yellow oil (161 g, 96%).

*6,6′-(6-Bromonaphthalen-2-ylazanediyl)dihexan-1-ol (**1**).* Into a 100 mL RBF containing 50 mL *tert*-butanol were added 6-bromonaphthalen-2-amine (22.2 g, 0.1 mol), 6-iodohexan-1-ol (50 g, 0.22 mol), and K_2CO_3 (30.4 g, 0.22 mol). After 96 h of reflux, the solution was filtrated, the solvent was removed under reduced pressure, and the crude product was purified on SiO_2 1:1 EE/heptane, to produce a pale brown oil (12 g, 28%).

*(E)-6,6′-(6-(2-(Pyridin-4-yl)vinyl)naphthalen-2-ylazanediyl)dihexan-1-ol (**2**).* To a solution of compound **1** (3.4 g, 8 mmol) in 30 mL of 2:1 TEA/DMF were added vinylpyridine (960 μL, 8.8 mmol), tris-(*o*-tolyl)phosphine (3 mol %), and $Pd(OAc)_2$ (3 mol %), after which the flask was flushed with argon, and the mixture was stirred at 120 °C for 120 m. Upon cooling, the solution was filtrated, and the solvents removed *in vacuo*. The crude products was purified on SiO_2 with 10:1 EE/heptane and then MeOH, to yield red needle-like crystals (2.2 g, 61%).

*(E)-3-(4-(2-(6-(Bis(6-hydroxyhexyl)amino)naphthalen-2-yl)vinyl)-pyridinium-1-yl)propane-1-sulfonate (**3**).* Compound **2** (1.4 g, 3.13 mmol), together with 3-bromopropane-1-sulfonic acid (1.4 g, 6.27 mmol), was dissolved in 140 mL of dry ACN and heated to 95 °C for 96 h under argon. The reaction mixture was cooled, the solvent was decanted off, and then the remaining brown solid was dissolved in MeOH, precipitated with ether, sonicated, and centrifuged. The red solid was washed 5 times with EE to remove the unreacted compound **2**, the product was washed from the remaining 3-bromopropane-1-sulfonic acid with 5 × 20 mL of EtOH, the fractions were pooled, and the solvent was removed under reduced pressure, yielding a black powder (765 mg, 43%).

*(E)-3-(4-(2-(6-(Bis(6-(methylsulfonyloxy)hexyl)amino)naphthalen-2-yl)vinyl)pyridinium-1-yl)propane-1-sulfonate (**4**).* A flask containing compound **3** (230 mg, 0.4 mmol) and methanesulfonyl anhydride (2.8 g, 16 mmol) was heated to 65 °C and stirred for 72 h. Upon cooling to RT, the mixture was dissolved in a minimal amount of dry DCM and mixed with 25 mL of ether. The suspended product was centrifuged down, and the pellet was dissolved and purified with 50:20:4 $CHCl_3$/MeOH/H_2O on SiO_2, producing a black powder (180 mg, 62%).

*(E)-3-(4-(2-(6-(Bis(6-(methyl(octyl)amino)hexyl)amino)naphthalen-2-yl)vinyl)pyridinium-1-yl)propane-1-sulfonate (**5**).* Compound **4** (400 mg, 0.6 mmol) and *N*-methyloctan-1-amine (687 mg, 4.8 mmol) were dissolved in 10 mL of ACN, the flask was flushed with argon, and the mixture was stirred at 50 °C for 72 h. The solvent was decanted off, and the mixture was dissolved and purified with 50:20:4 CH_2Cl_2/MeOH/H_2O on SiO_2 to yield a dark red tar (210 mg, 46.5%).

*(E)-3-(4-(2-(6-(Bis(6-(methyl(octyl)(phosphonooxymethyl)ammonio)hexyl)amino)naphthalen-2-yl)vinyl)pyridinium-1-yl)propane-1-sulfonate (**6**).* A solution of compound **5** (89 mg, 0.11 mmol), together with di-*tert*-butyl chloromethyl phosphate (284 mg, 1.1 mmol) and

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1,2,2,6,6-pentamethylpiperidine (198 μL, 1.1 mmol), in 2.3 mL of ACN was stirred under argon at 50 °C overnight. The solvents were removed under reduced pressure, 1 mL of 10% TFA in H_2O was added, and the mixture was stirred for 24 h. The TFA was removed under reduced pressure, and the mixture was basified with 3 drops of 8% NH_3, changing the solution from a pale orange to a dark red color. The product was purified on a Prontosil 120-20-C_{18} AQ (Bischoff) column with a 10 mmol ammonium acetate/ACN gradient and lyophilized to yield a dark red powder (4 mg, 3.5%, 2 steps).

Molecular Biology. The alkaline phosphatase gene, PLAP, was obtained from pORF14-PLAP (InvivoGen), and amplified *via* PCR using the primer pair 5′-TTGACCTCGAGATGATTCTGGGGCC-CTGCA-3′ and 5′-TTACGACGCGTTCAGGGAGCAGTGGCCG-TCTC-3′, to incorporate the restriction sites XhoI and MluI. The fragment was cloned into pIRES (BD Biosciences), to produce the vector pIRES-PLAP.

The H2B-GFP gene from pBOS-H2BGFP (BD Biosciences) was then cloned into this new plasmid *via* SalI and NotI to give pIRES-PLAP-H2BGFP. The expression vector was tested in the HEK293 cell line for GFP expression and for surface phosphatase activity, using the chromogenic NBT/BCIP Reagent Kit (Invitrogen). The functional PLAP-IRES-H2BGFP sequence was amplified using the primers 5′-TCTACTTAAGGCCACCATGATTCTGGGGCCCTGC-3′ and 5′-CTGCTCGAAGCATTAACCCTCAC-3′, introducing the AflI site at the 5′ end, and cloned into pcDNA5/FRT (Invitrogen) with AflI and NotI, producing pcDNA5FRT-IRES-PLAP-H2BGFP. Isogenic stable cell lines were produced *via* the cotransfection of pcDNA5FRT-IRES-PLAP-H2BGFP and the FLP-recombinase expression vector, pOGG44 (Invitrogen), in Flp-In-293 cells (Invitrogen). The FLP-induced, homologous recombination between the FRT sites in the vector and genome of the cells lead to the integration of the PLAP-IRES-H2BGFP construct and to hygromycin resistance, which was then used as a selection agent.

Cell Culture. Flp-In-293 cells were cultured using standard protocols in 35 mm polypropylene culture dishes (BD Biosciences), containing DMEM (high glucose), 10% FBS, 2 mM L-glutamine, 1% pen-strep, and 100 μg/mL zeocin, at 37.0 °C with 5% CO_2, and split using sterile PBS (containing no Ca^{2+} or Mg^{2+}) with trypsin-EDTA solution. The PLAP-IRES-H2BGFP stable cell line was cultured under the same conditions, with hygromycin replacing zeocin. For selective staining and voltage sensitivity experiments, both cell lines were split 1:20 into 35 mm dishes containing an uncoated 30 mm #1 glass coverslip (Thermo Scientific, Waltham, USA), using the described cell culture media without selection agents, and grown to 40% confluency.

Imaging. Images were taken with an iXonEM+ DU885KCS (Andor Technology), mounted on an Axiovert 135TV microscope (Carl Zeiss), with a Plan-NEOFLUAR 40x/1.30 oil-immersion objective. Fluorescence images were illuminated with Luxeon LEDs (Philips Lumileds); GFP images used the Luxeon V LXHL-LB5C (peak 470 nm), with an excitation bandpass filter (470/40 nm), and an emission filter (535/50 nm). VSD staining sequences used the same LED and excitation filter, with an emission long pass filter (590 nm). VSD sensitivity assays used the Luxeon V LXHL-LM5C (peak 530 nm), with an excitation filter (525/30 nm), and a long pass filter (610 nm) for emission. LED illumination was synchronized to camera acquisition timing to reduce bleaching.

Voltage Sensitivity. Extracellular voltages were applied to cultured cells in a custom chamber, consisting of a slit 18 mm long and 2.6 mm wide cut into the center of a 3 mm thick perspex disk. Platinum-wire electrodes, 0.5 mm diameter, were fixed against the long sides of the slit. A 30 mm coverslip with stained, cultured cells was placed face-up into a custom holder, and the perspex chamber pressed onto the coverslip and held in place by a threaded cylinder. The slit was then filled with buffer (as described below). The voltage patterns were generated by a 33120A Waveform Generator (Agilent), amplified by a custom-built push−pull circuit, and took the form of a square-wave pattern made up of 25 ms steps, ranging from 0 to ±5.5 V in 10 intervals. The LED illumination and voltage stimulus patterns were synchronized to the start of the camera acquisition sequence.

Protocols. For selective staining experiments, the culture medium was removed from dishes of mixed cell population on glass coverslips, and the cells gently rinsed once with wash buffer (100 mM NaCl, 70 mM D-glucose, 20 mM AMPD, pH 8.6), before the coverslip was transferred to a custom holder mounted to the microscope. The cells, covered by a thin film of wash buffer, were imaged under bright-field mode and for GFP fluorescence, and then the staining sequenced was recorded; 100 images were taken at 1 Hz, with an exposure time of 100 ms. After 5 images, 2 mL of staining buffer was gently added to the coverslip chamber (1 μM (*E*)-3-(4-(2-(6-(bis(6-(methyl(octyl)(phosphonooxymethyl)ammonio)hexyl)amino)naphthalen-2-yl)vinyl)pyridinium-1-yl)propane-1-sulfonate, 100 mM NaCl, 70 mM D-glucose, 20 mM AMPD, pH 8.6).

Voltage-sensitivity assays were performed on coverslips of stably transfected and untransfected control cells, washed once gently with wash buffer, and then stained for 120 s with 2 mL of staining buffer. The coverslips were then rinsed with wash buffer before being mounted into the voltage assay apparatus, and the chamber was filled with wash buffer. The image sequences of 32 images were recorded in frame transfer mode, with a combined exposure/read out time of 25 ms, synchronizing with the applied ramping spaced square-wave pattern.

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